Systematic analysis of longitudinal serological responses of pigs infected experimentally with African swine fever virus

Ana Luísa Reis,1 R. M. E. Parkhouse,1 Ana Raquel Penedos,1 Carlos Martins2 and Alexandre Leitão3

Correspondence
R. M. E. Parkhouse
parkhous@igc.gulbenkian.pt
Alexandre Leitão
alexandre@fmv.utl.pt

1Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal
2Laboratório de Doenças Infecciosas, CIISA, Faculdade de Medicina Veterinária, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal
3Instituto de Investigação Científica Tropical, CVZ, CIISA, Avenida da Universidade Técnica, 1300-477 Lisboa, Portugal

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The protective immune response to African swine fever virus (ASFV) includes both cellular and serological components. In this study, the role of antibodies in the pathogenicity and diagnosis of African swine fever (ASF) was explored. Accordingly, total and Ig isotype antibody responses against the 12 viral proteins previously demonstrated to be the main targets of serological immunity were evaluated in longitudinally collected sera from pigs infected experimentally with the non-pathogenic ASFV/NH/P68 isolate. Strong total IgG antibody responses were observed against viral proteins E183L/p54, K205R/‘unassigned’, A104R/histone-like and B602L/‘unassigned’; therefore, IgM, IgG1 and IgG2 responses to these proteins were also determined. One protein stimulating IgM (K205R) may have practical potential for the detection of recently infected animals. There was a clear trend towards an IgG1 response to all of the proteins. This may reflect a dominant Th2-controlled immune response. In order to identify possible correlations between these serological responses and the pathogenesis of ASF, total IgG responses to the 12 recombinant proteins were compared in asymptomatic and chronically infected animals. For the proteins NP419L/DNA ligase, CP312R, B646L/p73, K196R/thymidine kinase and K205R, the antibody titres were significantly higher in animals developing lesions. One exception was the antibody response to the A104R/histone-like protein, which was higher in asymptomatic than in chronically infected pigs, suggesting that antibodies against this protein might be an indicator of an effective immune response or that this response is somehow involved in protection.

INTRODUCTION

African swine fever virus (ASFV) is a large DNA virus and the only known member of the family Asfarviridae (Dixon et al., 2005). It is the aetiologic agent of a domestic pig disease that can assume different clinical forms, ranging from an acute haemorrhagic disease to inapparent infection. The virus is endemic in Sardinia and sub-Saharan countries, representing a serious problem to the development of the pig industry. Furthermore, with increasing infections in Africa and commercial trade among countries, the possibility of outbreaks in other countries cannot be excluded.

The development of a protective immune response occurs in the natural hosts (the warthog and the bush pig) and in domestic pigs that recover from the infection and are challenged with a homologus isolate (Malmquist, 1963). However, the mechanisms of this protective response are not entirely understood. Several reports have demonstrated an important role of antibodies in protective immunity through passive antibody transfer experiments (Wardley et al., 1985; Onisk et al., 1994). In addition, immunization with the viral structural proteins p32 and p54 (Gómez-Puertas et al., 1998) was found to be insufficient for protection, with 50% of immunized animals surviving a virulent virus challenge, although later developing clinical signs. Finally, in a more recent study (Neilan et al., 2004), the only observed effect of immunization with p32, p54 and p73 was a delayed onset of the disease. Clearly these results indicate that complete protection also requires a cellular component of the immune response. Indeed, ASFV-specific cytotoxic T lymphocytes able to lyse infected macrophages have been identified after infection with a non-haemadsorbing isolate (Martins et al., 1993), and the involvement of CD8+ T cells in protection was demonstrated recently (Oura et al., 2005). In addition, increased
natural killer cell activity was also found to be correlated with protection following inoculation with the ASFV/NH/P68 isolate (Leitão et al., 2001). Finally, multiple targets of both serological (Kollnberger et al., 2002) and cellular (Jenson et al., 2000) immune responses have been demonstrated.

This complexity of the protective immune response has thus impaired the development of an effective vaccine and studies directed towards understanding immunological correlates of the disease. Control is still based on diagnosis and the subsequent adoption of strict sanitary measures. There is therefore a requirement for rapid and reliable diagnostic procedures, preferably ones that do not involve the use of infectious virus.

Although the principal aim of this study was to explore the role of antibodies in pathogenesis, the results may also provide the basis for the future development of diagnostic tools that could improve the efficiency of outbreak control and the eradication of this disease in endemic countries. Thus, we analysed longitudinal antibody responses against the 12 viral proteins that have been demonstrated to be the main targets of serological immunity (Kollnberger et al., 2002) in pigs infected with the non-fatal, non-haemadsorbing ASFV/NH/P68 isolate. Infection of pigs with this isolate establishes protective immunity to subsequent challenge with the virulent Lisbon 60 ASFV isolate (Leitão et al., 2001) and results in a variable presence or absence of lesions. Strong and sustained antibody titres were observed against four of the 12 recombinant proteins tested; therefore, the longitudinal IgM, IgG1 and IgG2 responses to these proteins were also determined. The infected animals were further divided into two groups – those that were asymptomatic and those with clinical signs – in order to identify possible serological correlates of pathogenicity.

**METHODS**

**Sera.** Sera from pigs experimentally infected with the non-fatal, non-haemadsorbing ASFV/NH/P68 isolate have been described previously (Leitão et al., 2001). Sera used in this study comprised groups 3 and 4. The number of sera available at various days post-infection (p.i.) is presented in Table 1.

<table>
<thead>
<tr>
<th>Symptoms*</th>
<th>No. sera available at day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 4 7 11 14 21 28 39</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>5 10 5 5 10 10 5 5</td>
</tr>
<tr>
<td>Chronic disease</td>
<td>2 5 3 3 5 3 3 3</td>
</tr>
<tr>
<td>Total</td>
<td>7 15 8 8 15 15 8 8</td>
</tr>
</tbody>
</table>

*Symptoms of experimentally infected pigs from which sera originated.

**Table 1. Summary of experimentally infected pig sera used to evaluate the longitudinal antibody responses to ASFV recombinant proteins**

Cloning of the principal serological epitopes of ASFV. PCR primers containing specific restriction sites were designed for the viral open reading frames of the following proteins: A104R (histone-like), K78R (p10), CP204L (p32), E183L (p54), B646L (p73), F334L (ribonucleotide reductase), K196R (thymidine kinase), NP419L (DNA ligase), B602L, C44L, CP312R and K205R. The genes were amplified by PCR using BaTV isolate genomic DNA as template, and the PCR products were cloned into the pGEX-4T expression vector (Amersham). Cloned inserts were verified by sequence analysis and constructs were used to transform competent *Escherichia coli* strain BL21 (Stratagene).

**Protein purification.** Transformed *E. coli* cells were grown overnight in selective medium. The culture was diluted 1:50 in fresh Luria-Bertani medium and grown for 2 h. Expression of recombinant proteins was induced with 1 mM IPTG for 2 h and cells were harvested by centrifugation at 7500 r.p.m. in a Sorvall SLA-1500 rotor for 10 min.

Soluble proteins (histone-like, p10, p32, B602L, C44L, CP312R and K205R) were purified using RediPack GST Purification Modules (Amersham) according to the manufacturer’s protocol.

Insoluble proteins (p54, p73, ribonucleotide reductase, thymidine kinase and DNA ligase) were purified after solubilization with urea. Briefly, pellets were resuspended in 10 ml 50 mM Tris/HCl and lysozyme (1.25 mg) was added. The cells were lysed by several cycles of sonication, centrifuged and the pellet was washed five times in 5 ml 50 mM Tris/HCl with 1% Triton X-100. Next, 8 M urea (2.5 ml) was added and the mixture was sonicated until the pellet dissolved. The suspension was centrifuged at 11 000 r.p.m. in a Sorvall SS-34 rotor for 10 min. Urea was removed from the supernatant using an Amersham Biosciences PD-10 desalting column with PBS as the elution buffer.

**ELISA**

**Total IgG.** ELISA plates (Nunc) were coated with ASFV recombinant proteins (50 μl per well) diluted to the appropriate concentrations (1–10 μg ml⁻¹) in coating buffer (50 mM sodium carbonate/bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. The wells were then washed three times with PBS plus 0.05% Tween 20 and blocked with PBS plus 5% milk (200 μl per well) at 37°C during 1 h. After blocking, plates were washed five times as above and incubated for 1 h at 37°C with pig sera diluted 1:200 in PBS plus 5% milk (50 μl per well). The plates were again washed five times and incubated with protein A–horseradish peroxidase (HRP; Sigma) diluted 1:4000 (100 μl per well) for 1 h at 37°C. Finally, plates were washed again and developed with 0.5 mg o-phenylenediamine ml⁻¹ diluted in 0.2 M Na₂HPO₄, 0.1 M citric acid (pH 5.6) in the dark at room temperature for 15 min. After stopping the reaction with 0.1 M H₂SO₄ (50 μl per well), the A₄₅₀ was read on a Bio-Rad microplate reader.

**IgM/IgG1/IgG2.** ELISA plates were coated and blocked as described for total IgG. After washing, the plates were incubated for 6 h at room temperature with pig sera diluted 1:200 in PBS plus 5% milk (50 μl per well). The plates were then washed five times and incubated with mouse anti-porcine IgM (MCA637), IgG1 (MCA635) or IgG2 (MCA636; all from Serotec) diluted 1:250 (50 μl per well) overnight at 4°C. After incubation, plates were washed and incubated with HRP-conjugated rabbit anti-mouse Ig (Dako) diluted 1:1000 for 2 h at 37°C. Finally, the plates were developed and the absorbance measured as described above.

**Statistical analysis.** Statistical analysis of the results was carried out using repeated measures analysis of variance. Differences were considered to be statistically significant for values of *P*< 0.05.
RESULTS

IgG responses to ASFV recombinant proteins

In a previous study, the 12 principal serological epitopes of ASFV were identified by antibody screening of a cDNA library using serum from a pig that had survived infection with the virulent Malta isolate (Kollnberger et al., 2002). As this serum was chosen because of its high antibody titre, it could be presumed that not all of these proteins would necessarily elicit a response in all ASFV-infected animals. In order to identify serological correlates of pathogenicity and the virus proteins with potential as diagnostic targets, the 12 viral proteins were cloned and expressed, and the corresponding antibodies present in longitudinal sera from 15 pigs infected with a non-fatal isolate were defined by ELISA (see Methods).

The serological responses to the 12 recombinant ASFV proteins (Fig. 1) could be divided into three groups: group I with a strong antibody response, comprising E183L/p54, K205R/‘unassigned’, A104R/histone-like and B602L/‘unassigned’; group II with an intermediate antibody response, comprising B646L/p73, CP204L/p32, CP312R/‘unassigned’, NP419L/DNA ligase and F334L/ribonucleotide reductase; and group III with a poor antibody response, comprising K196R/thymidine kinase, K78R/p10 and C44L/‘unassigned’.

In group I, there was a clear increase in antibody titres on day 14 p.i., with 100% sensitivity when E183L/p54, K205R and A104R/histone-like proteins were used. In the case of B602L, the sensitivity reached 100% by day 21 p.i. In addition, the serological response to these proteins remained high at least until day 39 p.i. In group II, there was also an increase in antibody titres on day 14 p.i., but the sensitivity never reached 100%, even at later times p.i.

IgM responses to ASFV recombinant proteins

The proteins classified as group I were tested further by ELISA for their ability to detect recently infected animals.
through their early IgM responses (Fig. 2). In the majority of cases, IgM responses appeared at 7 days p.i. and started to decrease on day 14 p.i. When the K205R protein was used, seven out of eight animals could be identified as infected on day 7 p.i. Furthermore, antibody responses in all animals could be detected on day 11 p.i.

**IgG1 and IgG2 responses to ASFV recombinant proteins**

As IgG isotype expression is controlled by the different cytokines that polarize the immune response to Th1 (cell-associated) or Th2 (antibody-associated) immune responses (Stevens et al., 1988), the dominant IgG isotype reflects the dominant CD4 T-cell immune response. In order to identify the dominant IgG isotype response, group I proteins were tested in ELISAs, focusing on IgG1 and IgG2 antibodies. For all of the proteins tested, there was a clear trend towards the IgG1 response (Fig. 3), and this was particularly evident in the case of the E183L/p54 protein. Higher IgG1:IgG2 ratios were observed on day 11 p.i., with a decrease at later times. Nevertheless, on day 39 p.i., the dominant isotype was still IgG1.

**Antibody response in asymptomatic versus chronically infected pigs**

In order to identify possible correlations between the serological response to a specific protein and the pathogenesis of African swine fever (ASF), total IgG responses to the different ASFV recombinant proteins were compared in asymptomatic and chronically infected animals (Fig. 4).
For the proteins NP419L/DNA ligase, CP312R, B646L/p73, K196R/thymidine kinase and K205R, the antibody titres were significantly higher in animals that developed lesions. For the other proteins, no significant differences were observed between asymptomatic and chronically infected animals. However, in contrast to the 11 other recombinant virus proteins, the antibody response to the A104R/histone-like protein in asymptomatic animals was above that of chronically infected pigs. This observation was further confirmed when IgM (Fig. 5), IgG1 (Fig. 6a) and IgG2 (Fig. 6b) responses were evaluated.

No differences were observed in IgG1 : IgG2 ratios in asymptomatic versus chronically infected pigs (data not shown).

**DISCUSSION**

The immune response to ASFV is highly complex and includes both cellular and serological components. A systematic approach to defining the serological response of ASFV-infected animals might permit a better understanding of the role of antibodies in the disease pathogenesis. The first step towards this systematic evaluation was the identification of the 12 principal serological epitopes of ASFV through anti-ASFV antibody screening of an expression library constructed from ASFV-infected cells (Kollnberger et al., 2002). As these epitopes are recognized by both the domestic pig and the bush pig, an exploration of their immunogenicity in virus-infected pigs may contribute to our understanding of the pathogenesis of this disease and may also identify potential diagnostic tools.

In this report, sera from pigs infected with a non-fatal isolate that protects against subsequent challenge with the highly virulent L60 isolate (Leitão et al., 2001) were used to evaluate longitudinal antibody responses against these 12 recombinant viral proteins. Strong antibody responses...
were found to the proteins E183L/p54, K205R, A104R/histone-like and B602L, suggesting that they merited further investigation as possible diagnostic tools.

The expression of different IgG isotypes is controlled by cytokines secreted by CD4 cells. In the pig, B-cell responses to these cytokines in vitro are highly variable among Fig. 5. Longitudinal IgM responses in chronic (■) versus asymptomatic (●) pigs. ELISA plates coated with ASFV recombinant proteins were used to determine specific IgM responses at various days p.i. Results are presented as means ± SD of each group.

Fig. 6. Longitudinal IgG1 and IgG2 responses in chronic (■) versus asymptomatic (●) pigs. ELISA plates coated with ASFV recombinant proteins were used to determine specific IgG1 (a) and IgG2 (b) responses at various days p.i. Results are presented as means ± SD of each group.
animals, but the IgG2 isotype is considered to be associated with a Th1-cell-controlled immune response, whilst the IgG1 isotype is associated with a Th2-cell-controlled response (Crawley et al., 2003). In this context, the clear dominant IgG1 response to the four ASFV recombinant proteins observed in this report may reflect a dominant Th2-controlled antibody-mediated immune response. However, this observation does not necessarily imply that the Th2-mediated antibody response is the most effective in protection, as the development of chronic lesions was associated with a significant increase in antibody levels (Leitão et al., 2001). Porcine IgG isotypes were shown to have different biological functions (Crawley & Wilkie, 2003), and this was particularly evident in their ability to activate complement, with IgG2 being more effective than IgG1. As no differences were observed in IgG1:IgG2 ratios in asymptomatic versus chronically infected pigs, it could be concluded that the development of lesions cannot be attributed to differential expression and biological activity of IgG isotypes.

In order to identify possible differences in antibody responses of animals developing lesions and those remaining asymptomatic after infection with the ASFV/NH/P68 isolate, total IgG responses to the different ASFV recombinant proteins were compared in these animals. The higher antibody titres observed in chronically infected animals for the proteins NP419L/DNA ligase, CP312R, B646L/p73, K196R/thymidine kinase and K205R probably reflect the late viraemias that correlate with the occurrence of lesions in the model of protection used in this work (Leitão et al., 2001). The antibody response in asymptomatic animals was only higher than that of chronically infected pigs with one recombinant protein (A104R/histone-like protein). However, no statistical significance was found for this result. Nevertheless, the same trend was observed when IgM, IgG1 and IgG2 responses were evaluated, suggesting that antibodies produced against this protein might indicate that an effective immune response has taken place or perhaps that the response is involved in protection. The histone-like protein is thought to be associated with the viral DNA (Neilan et al., 1993) and is therefore not a logical target for antibodies mediating virus neutralization or complement-mediated destruction. Clearly, further work is necessary to define the possible role of this protein in pathogenesis.

Several studies have focused on the antibody responses to the proteins described as containing neutralizing epitopes of the virus (p32, p54 and p73) (Zsak et al., 1993; Gómez-Puertas et al., 1996, 1998; Neilan et al., 2004). However, no consistent sterilizing immunity was found after immunization with these proteins. Surprisingly, and in view of the possible participation of antibodies in protection (see above and Introduction), the responses to the proteins containing neutralizing epitopes of ASFV, p73 and p32 (Zsak et al., 1993; Gómez-Puertas et al., 1996, 1998), were variable, with three out of the 15 animals not having detectable antibodies against these proteins on day 21 p.i. In addition, in the case of the p73 protein, the antibody titres were significantly higher in chronically infected animals, suggesting that antibodies directed to this capsid protein may not protect against the development of clinical signs. This significant result cannot be ascribed to high titres of virus acting as an immunoadsorbant to deplete levels of circulating antibodies. As published previously, of the 240 blood samples collected at different times after virus inoculation in this study, virus was detected, albeit at low titre (<10^3 CPE50/ml), in only 36 samples (Leitão et al., 2001). Unfortunately, in our ELISAs there was high background with p32 protein (exclusive for this protein), which did not allow a good discrimination between infected and non-infected pig sera. In another study using p32 produced in the baculovirus system, good sensitivity was reported for detection of infected animals (Pérez-Filgueira et al., 2006).

Diagnosis of ASF is conventionally carried out by direct immunofluorescence and virus isolation using primary pig leukocyte cultures and visualization of haemadsorption (OIE, 2004), and thus requires dedicated isolation laboratories and the routine preparation of primary leukocyte cultures. In endemic areas, diagnosis is based on the detection of antibodies, generally using infected cells as a source of viral antigens, which is potentially hazardous and also requires biosafety facilities. Thus, identification of the ASFV immunodeterminants eliciting a strong and sustained antibody response is a step towards the development of more sensitive and specific serological diagnostic tools.

Measurement of serum antibody to recombinant protein would be a reproducible and safe alternative to the methods described above, allowing the standardization of antigen production and eliminating the need for infectious material manipulation. Several studies focusing on the use of recombinant ASFV proteins for serological diagnosis have shown promising results (Oviedo et al., 1997; Barderas et al., 2000, Gallardo et al., 2006; Pérez-Filgueira et al., 2006). However, given the high variability of ASFV isolates in Africa, high specificity and sensitivity may only be achieved by the use of more than one protein. For example, ELISAs using p32 as antigen showed a marked decrease in sensitivity when sera from Eastern Africa were used, an observation that has been suggested to correlate with p32 antigenic variation (Pérez-Filgueira et al., 2006). Semi-purified p73 was described as giving good results when used for the detection of antibodies by ELISA (Vidal et al., 1997). However, the results obtained here, using bacterially expressed recombinant p73, indicated that this protein may not be a good target for serological assays, as it failed to detect carriers with inapparent symptoms.

In this report, four ASFV recombinant proteins (E183L/p54, K205R, A104R/histone-like and B602L) were identified as potential serological diagnostic antigens, all showing 100% sensitivity at 21 days p.i. Whether or not any of these four candidate proteins identified in this study will prove to be adequate diagnostic tools will depend on...
extensive validation with appropriate panels of sera. The possibility of any of these four proteins demonstrating a highly variable reactivity with different ASFV isolates, however, is unlikely, as the antigenicity/hydrophobicity plots of these four proteins from all sequenced isolates (Preto-risuskop-96-4, MalawiLil-20-1 1983, Kenya 1950, Mkuzi 1979, Ba71V, Tenganí 62, Warthog and Warmbath; http://athena.bioc.uvic.ca/database.php?item=listGenomes&db=asfarviridae) are strikingly similar. Indeed, in one case (A104R), there was only one amino acid difference among the different isolates. A pertinent point is that the antibody response of the pig is, of course, polyclonal and is thus unlikely to focus on one of the many antigenic epitopes of a given viral protein.

During outbreaks of ASF, rapid and reliable diagnosis is a key point in disease control. Several PCR-based ASF diagnosis assays have been described (Aguero et al., 2003; Hjertner et al., 2005; Zsak et al., 2005) that permit the detection of early stages of infection. Nevertheless, the development of ELISAs able to identify recently infected animals would be useful, as this technique allows the simultaneous handling of multiple samples, has less risk of cross-contamination and the time-consuming step of DNA extraction from tissues is not necessary. In order to identify appropriate antigens for such ELISAs, the four antigens showing the strongest antibody responses were tested further for their ability to detect recently infected animals through IgM responses. One protein (K205R) was identified as a potentially useful serological diagnostic antigen for the detection of IgM responses, as it was able to detect all infected animals at 11 days p.i. In addition, the majority of infected animals could be identified as early as 7 days p.i. when this protein was used. More extensive validation of this protein is clearly indicated.

The determination of IgM titres would also be useful for epidemiological studies in endemic areas, as positive detection of antibodies indicates previous exposure and not necessarily a current infection. In this case, the IgM:IgG ratio would distinguish between exposed and recently infected animals, as there was a marked increase in IgG levels on day 14 p.i. followed by a marked decrease in IgM levels.

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